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High serum levels of proinflammatory markers during epileptogenesis. Can omega-3 fatty acid administration reduce this process?

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ABSTRACT

During the epileptogenic process, several events may occur, such as an important activation of the immune system in the central nervous system. The response to seizure activity results in an inflammation in the brain as well as in the periphery. Moreover, CRP and cytokines may be able to interact with numerous ligands in response to cardiac injury caused by sympathetic stimulation in ictal and postictal states. Based on this, we measured the serum levels of C-reactive protein (CRP) and cytokines during acute, silent, and chronic phases of rats submitted to the pilocarpine model of epilepsy. We have also analyzed the effect of a chronic treatment of these rats with omega-3 fatty acid in CRP and cytokine levels, during an epileptic focus generation. C-reactive protein and cytokines such as IL-1β, IL-6, and TNF-α presented high concentration in the blood of rats, even well after the occurrence of SE. We found reduced levels of CRP and all proinflammatory cytokines in the blood of animals with chronic seizures, treated with omega-3, when compared with those treated with vehicle solution. Taken together, our results strongly suggest that the omega-3 is an effective treatment to prevent SUDEP occurrence due to its capability to act as an anti-inflammatory compound, reducing the systemic inflammatory parameters altered by seizures.

1. Introduction

Several lines of evidence have shown the involvement of inflammatory pathway with temporal lobe epilepsy (TLE) [1–3]. Patients with specific forms of epilepsy present with a peripheral inflammatory response linked to the condition; furthermore, an increased cytokine response has also been reported in the literature in these cases [4].

Another event that occurs during long-lasting seizures is dysfunction of the blood–brain barrier (BBB) [5,6], allowing the entrance of proteins and cells from the blood into the brain, increasing local inflammation, and amplifying excitability in a continuous cycle. In this context, lymphocytic infiltrate and high cytokine levels suggest that both humoral and cellular immune systems are exacerbated in these patients [2].

C-reactive protein is an important molecule related to the acute phase of the inflammatory process; it is present in the serum or plasma of many vertebrates [7]. Its main effect is the modulation of several cascades related to immune system activation [8]. It has been linked to inflammatory processes, it has been used clinically as a marker for immune responses to infections, and it has an important biological role in the pathogenesis of cardiovascular disease [9]. Transcriptional regulation of CRP has been extensively studied *in vitro* and *in vivo*. For this regulation, several evidences suggest that IL-6 is the principal inducer of the CRP gene expression, while IL-1, TNF-α, glucocorticoids, and activated complement act synergistically with IL-6 to enhance its effect [10–12]. An increased CRP level in the blood is considered to be an inflammatory marker for brain ischemia, stroke, and vascular events [13].

A few studies provided clinical and experimental evidence suggesting that omega-3 fatty acid supplementation decreases the duration and frequency of seizures [14–16], resulting in neuroprotective effects against seizure-induced brain damage [17]. In patients with refractory seizures, it has been suggested, though not proven, that treatment with omega-3 fatty acid could also reduce seizure-associated cardiac arrhythmias and, in some cases, sudden unexpected death (SUDEP), the most important direct epilepsy-related cause of death [18,19].

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Thus, it becomes necessary to better understand the role played by the immune system in epilepsy; therefore, the main objective of this study was to investigate CRP, IL-1β, TNF-α, IL-6, and IL-10 levels in the blood of rats submitted to the pilocarpine of temporal lobe epilepsy. This model consists of an acute phase, characterized by long-lasting *status epilepticus* (SE) (12 h), followed by a silent period with no seizures (lasting 4 to 44 days) culminating in a chronic phase (period of spontaneous seizures), lasting for the rest of the animal's life [20]. We also analyzed the levels of these immunological markers in the blood of epileptic rats (chronic phase), daily treated with omega-3 fatty acid, after SE onset.

2. Material and methods

2.1. Animals and status epilepticus induction

The animal experiments were performed under UNIFESP Institutional Ethical Committee approval (Hospital São Paulo/Universidade Federal de São Paulo, process n. 313162), and all efforts were made to minimize animal suffering. Wistar adult male rats, weighing 250 g, were housed in groups of three to four per cage and maintained in controlled room temperature, humidity, and light–dark cycle (12:12 h) with chow pellets and tap water available *ad libitum*.

The rats received a single dose of pilocarpine (350 mg/kg, intraperitoneal [i.p.]). To prevent peripheral cholinergic effects, scopolamine methylnitrate was injected subcutaneously at a dose of 1 mg/kg, 30 min before pilocarpine administration.

To stop *status epilepticus* (SE) during the acute phase of the pilocarpine model, diazepam (10 mg/kg, Cristalia-Compaz) was administered subcutaneously 3 h after SE onset. The animals were then allowed to evolve from the acute to the silent and chronic phases of this model as previously reported by us [20]. The occurrence of spontaneous recurrent seizures (SRSs) during the chronic period was video-monitored (24 h per day) for 90 days. Animals were sacrificed, and the blood was collected for CRP and cytokine assay.

2.2. Animal groups

2.2.1. C-reactive protein

To study the influence of long-lasting SE or spontaneous seizures (chronic period) on peripheral C-reactive protein, the following groups (n = 8) were analyzed: 5 h, 12 h, and 24 h after SE onset (acute period); 48 h and 5 days after SE induction (silent period), and 90 days after SE onset (chronic phase, period of spontaneous seizures). Saline-treated animals were used as controls for each group (n = 8) as well as rats that received pilocarpine but did not develop SE.

2.2.2. Cytokines

To study the inflammatory profile (IL-1β, TNF-α, IL-6, IL-10) in the blood, the following groups were performed: acute (5 h), silent (5 days), and chronic (90 days after SE onset). These groups were compared with saline-treated rats (n = 6 each group).

2.2.3. Omega-3 treatment

Rats were submitted to SE, which was blocked with diazepam 3 h after onset. After that, animals received vehicle cremophor (0.009%) or fish oil (omega-3, PROEPA, 85 mg/kg/day). These solutions were administered to animals between 11:00 and 12:00 am by gavage. The volume administered was adjusted according to animal weight, which was verified three times a week, for 90 days. Omega-3 fatty acid was formulated as fish oil (EPA 180 mg and DHA 120 mg). The capsule contents were dissolved in cremophor 0.009%, yielding a final concentration of 21.25 mg/ml fish oil, which corresponds to 3.82 mg/ml EPA and 2.55 mg/ml DHA. At the final concentration, fish oil was administered 1 ml per 250 g of animal weight. Animals were killed by decapitation, and serum was collected and stored at −80 °C for CRP and cytokine analyses. Four groups were analyzed: control vehicle (animals which received saline and cremophor), control omega (animals which received saline and omega-3), chronic (animals with epilepsy, 90 days after SE induction), chronic + omega (animals with epilepsy which received omega-3 treatment) (n = 6 for each group).

2.3. Measurement of inflammatory markers

2.3.1. CRP assay

Quantitative assessment of CRP levels was carried out *via* ELISA (C-reactive protein, ELISA kit, Chemicon, Millipore, MA, USA), following the manufacturer's recommendations.

2.3.2. Cytokine analysis

Multiplex immunobead assay technology (MAP Milliplex Rat Cytokine/Chemokine Magnetic Bead Panel Millipore Corp., Billerica, MA, USA; Magpix and analytical test instrument, Luminex Corp., Austin, TX, USA) was performed in serum.

2.4. Statistical analysis

The SPSS statistical package version 22.0 (SPSS, Chicago, IL, USA) and GraphPad version 6.0 were used for statistical evaluation (GraphPad Software, San Diego, CA, USA). Data are expressed as the mean ± standard deviation (SD). Given the sample size and the variable distribution, nonparametric tests were used. Data for three or more independent groups were analyzed by Kruskal–Wallis test. When significant, a multiple comparison *post hoc* test was used (Dunn's test). A two-tailed p-value < 0.05 was chosen as the level of significance. Statistical data are provided in the figures.

3. Results

3.1. Animal behavior

Pilocarpine administration induced the following behavioral changes: akinesia, facial automatisms, and limbic seizures consisting of forelimb clonus with rearing, salivation, and masticatory jaw movements and falling. This type of behavior built up progressively into motor limbic seizures that recurred repeatedly, evolving to long-lasting *SE* as previously reported [21].

3.2. CRP levels

Control rats presented CRP serum levels ranging from 6.8 ± 1.5 to 10.3 ± 3.3 ng/ml. However, after SE induction, the CRP concentration rose drastically mainly after 5 h of SE. During the silent phase, CRP levels were still high within 2 days after SE and remained higher 5 days after SE onset. Animals presenting spontaneous seizures (90 days after SE) showed high CRP concentration, when compared with control levels. The comparison between all groups is presented in Fig. 1A, which shows that CRP elevation is not restricted to seizure period. Its level remained altered in all periods of the epilepsy model induced by pilocarpine.

There were no differences in CRP levels between controls treated with vehicle and controls treated with omega-3 fatty acid. Chronic rats (with spontaneous recurrent seizures) without treatment presented high values, when compared with its proper control groups. In contrast, chronic rats treated with omega-3 for 90 days after SE onset showed decreased levels of this inflammatory marker, when compared with the chronic group. The treatment with omega-3 fatty acid induced a reduction in CRP levels, but the treatment was not enough to bring CRP to normal levels. Treated rats showed CRP levels similar to those values found in animals during the silent period of this epilepsy model (5 days after SE) (Fig. 1B).

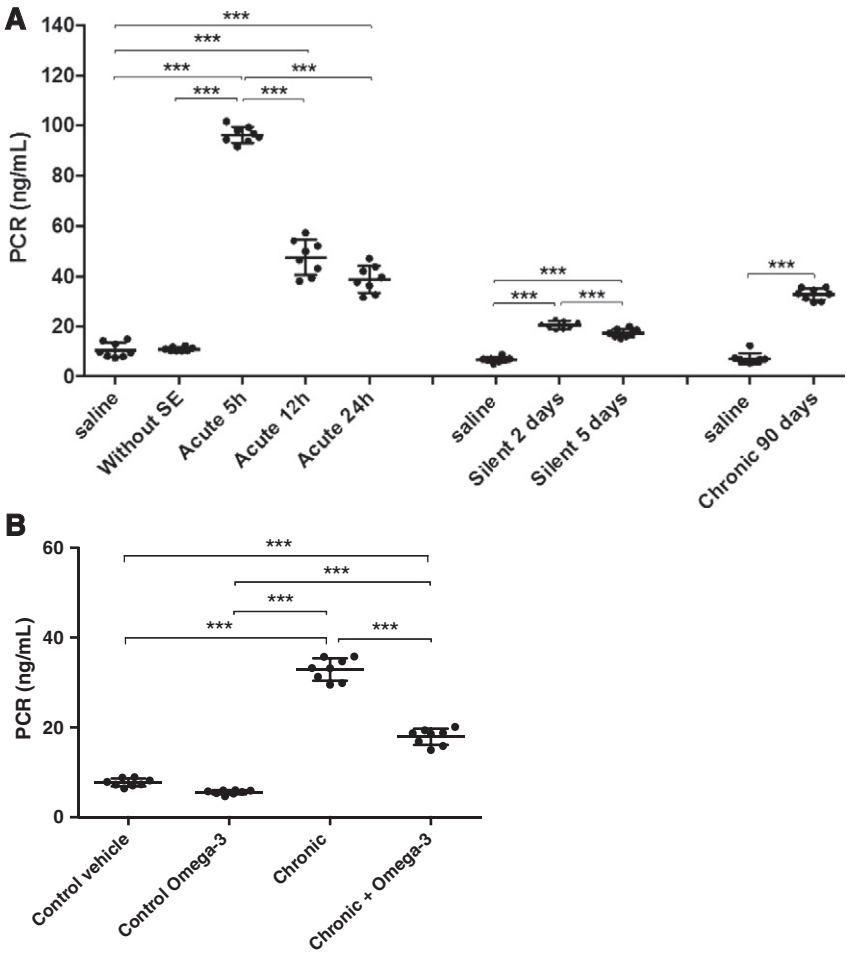

Fig. 1. A: Plasma C-reactive protein concentrations measured by ELISA during all periods of the pilocarpine model of epilepsy. In the acute phase, a significant increase was observed, mainly after 5 h; however, this augmentation remained higher after 12 and 24 h when compared with the saline-treated group. In silent period, the levels of CRP were still high, when compared with the saline-treated group. Interestingly, 90 days after SE, when animals presented spontaneous recurrent seizures, these levels increased again, when compared with the saline-treated group. ***$p < 0.001$. Mean ± SD. B. Plasma C-reactive protein concentrations measured by ELISA during chronic period of the pilocarpine model of epilepsy after omega-3 treatment. Note the decreased levels of plasma C-reactive protein after omega-3 treatment in the chronic + omega 3 group, when compared with the chronic group that were untreated group. ***$p < 0.001$. Mean ± SD.

According to these findings, it is clear that epileptic animals have increased CRP concentration in the blood, when compared with the control group, and that the treatment with fish oil reduced its levels.

3.3. Cytokine multiplex analysis

Multiplex analysis was performed by measuring the cytokines as IL-1β, TNF-α, IL-6, and IL-10 in plasma.

There were increased levels of IL-1β in the 5-hour ($p < 0.05$) and 90-day groups ($p < 0.001$). The 5-day group did not present any difference in plasma IL-1β levels when compared with the saline group (Fig. 2A).

Plasma TNF-α levels were higher in the 5-day and 90-day groups (Fig. 2B). The 5-hour group did not show a statistical difference when compared with the saline-treated group.

The IL-6 analysis showed a significant increase in all periods of the pilocarpine model, especially after 5 days of SE onset (Fig. 2C).

Interleukin-10, a key anti-inflammatory cytokine, started to increase 5 days after SE onset, and a significant increase was observed in the 90-day group (chronic period, $p < 0.001$) when compared with other groups and the saline-treated group (Fig. 2D).

After omega-3 treatment, plasma levels of all cytokines were reduced when compared with the chronic group (rats that exhibited a spontaneous recurrent seizure without omega-3 treatment) (Fig. 3A, B, C and D). Thus, these results showed an anti-inflammatory effect of this treatment. Rats that received pilocarpine but did not develop SE showed normal levels of CRP and cytokines (data not shown).

4. Discussion

The present work shows that during the epileptogenic process, the peripheral immune system is activated. C-reactive protein and cytokines such as IL-1β, IL-6, and TNF-α presented high concentrations in the blood of rats, even well after the occurrence of SE. These data are consistent with a relationship between peripheral mediators of inflammation and brain excitability. Previous reports have indicated that in the central nervous system (CNS), cytokines are produced as a response to various inflammatory stimuli and this production may be induced also by seizure activity [22,23].

Elevated levels of these inflammatory mediators in blood were not due to a possible long-term effect of pilocarpine, as a cholinergic agent, since we did not observe an increase in CRP levels in the serum of rats in the group without SE. According to Marchi et al. [24], pilocarpine may activate proinflammatory mechanisms and have direct action on CNS neurons, increasing brain excitability. This effect of pilocarpine inducing an increase in inflammatory markers was not observed here,

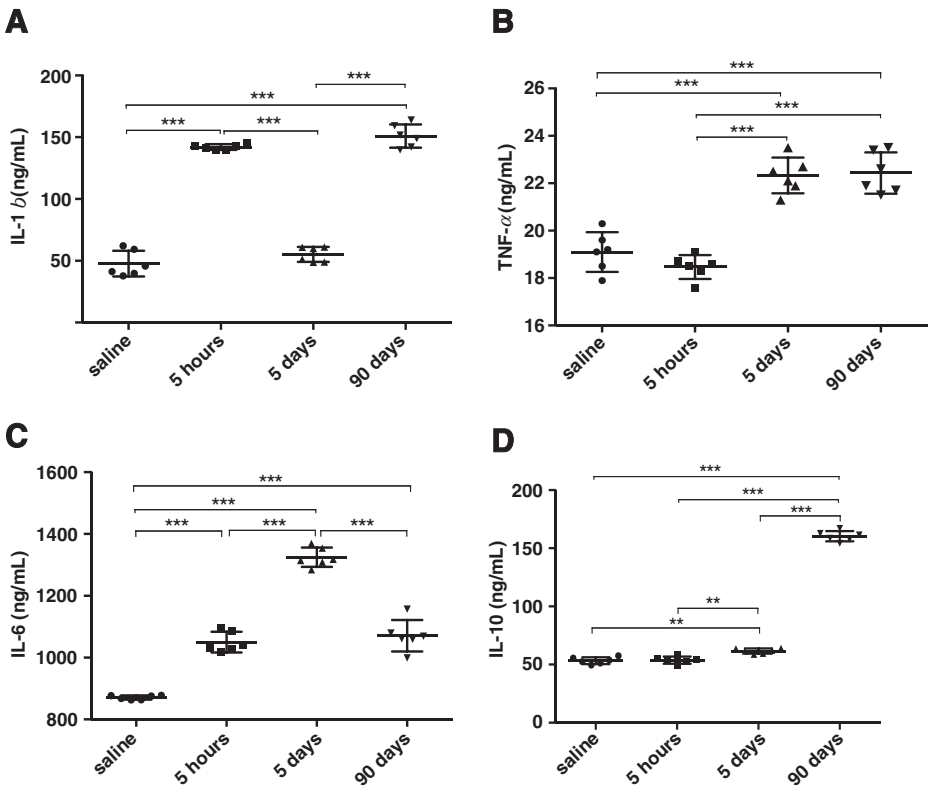

Fig. 2. Plasma cytokine concentrations measured by multiplex analysis. A: IL-1β levels were increased in the 5-hour (acute period) and 90-day groups (chronic period), showing the effect of seizure occurrence on IL-1β levels. B. TNF-α presented an increased level after 5 days in silent period and remained higher after 90 days (chronic period) C. IL-6 started to increase after 5 h and level was increased after 5 days in silent period and remained higher in chronic period (90 days after SE). D: IL-10 started to increase after 5 days (acute group) and presented higher level after 90 days (chronic group). All groups were compared with the saline-treated group. $^{**}p < 0.01$, and $^{***}p < 0.001$.

in contrast to Marchi's report. Furthermore, animals in the chronic phase, well after exposure to pilocarpine, also showed high levels of these inflammatory mediators.

Our data also indicated that treatment with omega-3 fatty acids, beginning close to a brain insult (SE), could reduce blood CRP and cytokine levels, demonstrating that these fatty acids may reduce inflammation after SE onset and during epileptogenesis.

According to Li et al. [5], seizures cause blood–brain barrier (BBB) disruption, characterized by the invasion of leukocytes into the brain. Danjo et al. [6] observed a loss of BBB integrity due to pentylenetetrazole (PTZ)-induced seizures. These authors reported that BBB disruption occurs due to high NO concentration, generated by neural nitric oxide synthase. These data were previously confirmed by our group [2] when we observed high levels of NO and lymphocyte infiltrates into the brains of patients with TLE. Moreover, CRP action is related to the modulation of polymorphonuclear cell function, complement system activation, and the activation of phagocytosis [8]. With BBB disruption, CRP from blood can enter into the brain, increasing the inflammatory process, which is well-documented by several authors [25–28]. This process triggers a vicious cycle in the brain since it is known that the inflammatory response affects the generation of seizures as well as seizure severity [29].

An important finding of this study is the reduced levels of CRP and all proinflammatory cytokines in blood of animals with chronic seizures, treated with omega-3, when compared with those treated with vehicle solution.

In addition, there is interest in the possible role of omega-3 fatty acid as a potential agent in reducing the risk of SUDEP, since previous data from our laboratory have demonstrated increased IL-6 levels in the hearts of animals submitted to the pilocarpine model of epilepsy [30]. Omega-3 fatty acid reduces sodium and calcium flux across excitable membranes, reduces hippocampal excitability [31], and improves cognitive performance after seizures in rats. It has been shown that omega-3 fatty acids have protective effects, preventing coronary heart disease, reducing arrhythmias and thrombosis, lowering plasma triglyceride levels, and reducing blood clotting tendency. Furthermore, substantial evidence from epidemiological and case–control studies indicates that omega-3 reduces the risk of cardiovascular mortality [32]. Because SUDEP is thought to occur during or shortly after a seizure, Taha et al. [33] have proposed that omega-3 fatty acids may reduce the incidence of SUDEP through their anticonvulsant effects. Using the pilocarpine model of epilepsy, we have shown that chronic omega-3 administration promotes neuroprotection and neuronal plasticity [17].

The occurrence of SUDEP has been associated with multifactorial mechanisms, but a number of interictal, ictal, and postmortem cardiac abnormalities account for the possibility of seizure-induced cardiogenic SUDEP [34,35]. Our group showed that rats with epilepsy have a higher resting heart rate than control rats [36], and a higher heart rate is a major independent risk factor for cardiovascular death [37]. Basing on this, the exact pathophysiology of SUDEP remains unknown, and one of the best strategies to minimize the risk of SUDEP is to establish preventive measures [38]. Thus, if cardiovascular abnormalities during and between seizures are directly related to a high frequency of SUDEP, treatment with omega-3 appears to be a potential SUDEP-prevention tool since substantial epidemiological evidence and case–control and experimental studies indicate the beneficial effects of omega-3 consumption on the cardiovascular system and the CNS and even in enhancing global quality of life of individuals with epilepsy [39].

CRP and proinflammatory cytokines are able to interact with numerous ligands associated with tissue response to cardiac injury and play an important role in the pathogenesis of cardiovascular disease [9], and ictal and postictal states are marked by sympathetic stimulation which affects cardiopulmonary function [40,41]. Moreover, the peripheral

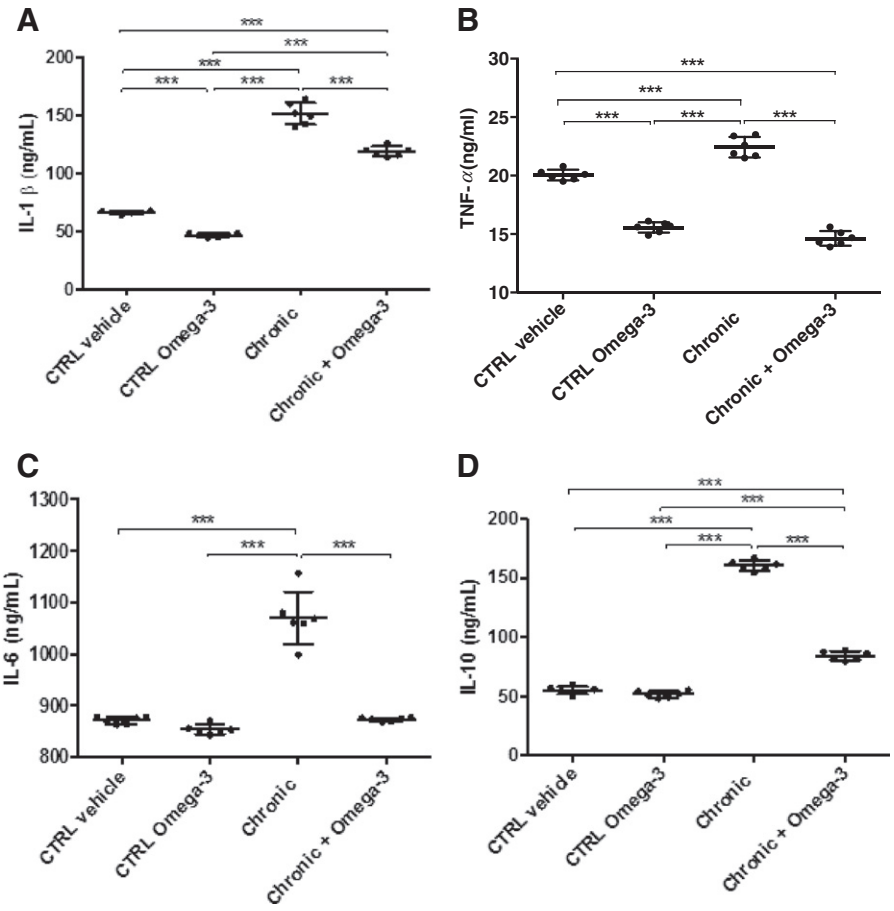

Fig. 3. Plasma cytokine concentrations measured by multiplex analysis after omega-3 treatment in the chronic period. All cytokines (IL-1β, IL-6, TNF-αI, and L-10) that showed increased levels in plasma during chronic period were reduced after omega-3 treatment when compared with the chronic group. ***$p < 0.001$.

markers are extremely important in monitoring the patient's state, and the establishment of treatment parameters is imperative. According to this, through the utilization of animal models, we can observe if omega-3 treatment was effective as anti-inflammatory therapy for epilepsy, mainly in order to try decreasing the SUDEP occurrence. However, future studies need to be done to evaluate the effect of omega-3 fatty acid administration in association with antiepileptic drugs on frequency and severity of seizures, as well as in cardiac remodeling parameters in people with refractory epilepsy.

Conflict of interest

There is no conflict of interest.

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